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ISOLATION OF AnsB GENE FRAGMENT ENCODING L-ASPARAGINASE 2 ENZYME FROM *Serratia plymuthica* UBCF_13 AND IT'S IN-SILICO DOMAIN CHARACTERISTIC

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ABSTRACT

Acute lymphoblastic leukemia (ALL) is one of the cancer diseases that often occurs in children and causes high mortality in children. One of the chemotherapy treatments suggested is using L-Asparaginase 2. However, due to its complex production process, this approach is expensive for the public. Therefore, the production technology of this enzyme is crucial, enabling cheaper treatments for ALL. This study aimed to isolate the AnsB gene sequence from Serratia plymuthica UBCF_13 and perform further in-silico analysis. The research was started by designing specific primers for the AnsB gene, isolating the AnsB gene fragment using a PCR-based approach, sequencing the AnsB gene fragment, cloning it to the plasmid vector, and further transforming it into E—coli DH5α cell. Further data was analyzed using bioinformatics tools such as BLAST, MEGA X, and I-TASSER InterPro. Sequence data results successfully verified that the full length of the AnsB gene is 1047 bp. InterPro analysis indicated that the L-Asparaginase 2 from S. plymuthica UBCF_13 has two domains, namely L-Asparaginase N-terminal spanning from amino acid 26 to 216, while its C-terminal spanned from amino acid 235 to 345. The physical fragment of the gene was also successfully cloned to the pGEM-T Easy vector and subsequently transformed into E. coli DH5α cell. This result provided information for alternative sources of L-Asparaginase 2 and its possible engineering.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is one of the cancer diseases that often occurs in children and causes high mortality in children. The death rate of this disease worldwide is ranked 11th, with the prevalence ranking 15th among all types of cancer. In 2030, it is estimated that there will be a significant increase in the incidence of leukemia, predicted up to 9.21 million people (Du et al., 2022). One of the recommended treatments for ALL includes chemotherapy using L-Asparaginase type 2 enzyme (Maese & Rau, 2022). The use of L-asparaginase enzyme type 2 has been approved by the Food and Drug Administration (FDA) (Nag et al., 2023). However, due to its high-cost production, the treatment is still expensive to implement for ALL patients. Therefore, alternative technology and sources of such enzymes are crucial to be obtained.

L-Asparaginase type 2 enzyme is naturally produced by various bacteria, fungi and other microbes. The current source is *Escherichia coli*, *Lactobacillus casei*, and *Erwinia chrysanthemi* (Darvishi et al., 2022). Aisyah et al. (2017) successfully isolated *S. plymuthica* strain UBCF_13 from *Brassica juncea* ***L.***, a vegetable grown in West Sumatra. Further study of this bacterium using Next generation Sequencing (NGS) technology has successfully revealed its complete genome sequence data and now is available at the NCBI database (Fatiah et al., 2021). Genome mining analysis indicated that this strain could produce the L- Asparaginase 2 enzyme, represented by the AsnB gene sequence along its whole genome. This data promises an alternative source for the L- Asparaginase type 2 enzyme productions.

In order to better understand its potential and engineering possibility for improvement of its activity, characteristics of the gene and its production conditions have to be further studied. In the first step for such activity, the availability of physical fragments of the gene and its characteristics is an important necessity. Therefore, isolation of the physical gene and subsequently cloning it to the bacterial cell for its conservation and further analysis must be performed before it. Based on the above-mentioned background, this study is purposed to isolate and clone the physical AsnB gene fragment from *S. plymuthica* strain UBCF_13 to the cloning vector pGEM-T Easy plasmid and finally transform it into *E. coli* strain DH5α cell. Another objective of this study is to characterize the L- Asparaginase 2 polypeptide sequence via in-silico analysis.

MATERIALS AND METHODS

Materials

The primary material used in this study is *S. plymuthica* strain UBCF_13 bacterial cells glycerol stock, which was previously isolated by Aisyah et al. (2017). This isolate is a collection of the Biotechnology Laboratory of Andalas University, Padang. *E coli* strain DH5α, plasmid pGem-T Easy vector (Promega-USA) were provided commercially from the appropriate producer.

Isolation of S. plymuthica UBCF_13 genomic DNA

The isolation of S. plymuthica UBCF_13 genomic DNA was performed using the GeneJET Genomic DNA Purification Kit (Promega, USA) as instructed by the company. The procedure was done as follows. The bacterial cell was cultured in a 1.5 ml microtube in a specific growth medium and under specific conditions, and the pellet was harvested by centrifugation at 5,000 rpm for 5 minutes. The pellet was treated with 200 µl of Lysis Solution and 20 µl of Proteinase K, and the vortexed. The suspension was then transferred to the purification column and centrifuged at 8,000 rpm for 1 minute. A 500 µl of Wash Buffer I was added to the purification column, then centrifuged at 8,000 rpm for 1 minute, followed by another 500 µl of Wash Buffer II. An elution buffer of 50 µl was added to harvest the DNA. The isolated DNA was then checked using electrophoresis in 1% agarose.

Specific primer designing for S. plymuthica UBCF_13 AnsB gene, a gene of particular interest in this study due to its role in [specific role or function]

A pair of specific primers were designed in order to amplify only the AnsB gene fragment from *S. plymuthica* UBCF_13 genomic DNA. The primer characteristics were developed with a thorough analysis, considering the GC composition and analyzing other factors using the Multiple Primer Analyzer at https://www.thermofisher.com/id/en/home.html. The primer's probability of hairpin structure formation was also assessed using the OligoCalc web tool at http://biotools.nubic.northwestern.edu/. The primer was designed based on the reference sequence retrieved from the NCBI database (accession code CP068771.1) located at bases 1517615 to 1518661. The appropriate annealing temperature of the designed primers was carried out using a gradient PCR program (Table 1). A range of annealing temperatures

was tested from 52 °C to 65 °C using the KOD-Plus-Neo Master mix (Table 1). The target expected amplicon size is 1,047 bp.

Table 1. PCR condition for gradient analysis.

Step	Temperature (°C)	Time (second)
Pra-denaturation	95	180
Denaturation	95	30
Annealing	50 – 60	30
Extension	72	120
Final extension	72	600
Pause	16	∞

Table 2. Cocktail composition for PCR gradient.

Component	Volume (µL)
KOD-Plus-Neo	6.3
Primer AnsB Forward (10 ng/µl)	1.0
Primer AnsB Reverse (10 ng/µl)	1.0
DNA *S. plymuthica* UBCF_13 (10 ng/µl)	1.0
Nuclease Free Water	3.2
Total volume	12.5

Sequencing and bioinformatic analysis

Sequencing of PCR product was performed by 1st Base Singapore using the Sanger method, a widely used technique for DNA sequencing, from both end terminals. The obtained sequence data were edited and assembled as a single contig from both directions. The verified consensus sequence was stored in FASTA format and used for BLAST and further analysis.

Ligation and transformation of the AnsB gene frame

The AnsB gene fragment, a key component in our study, was ligated into the pGEM-T Easy vector as recommended by the company (Promega, USA). This ligation process is crucial as it allows us to insert the gene fragment into the vector, which can then be used to transform E. coli cells for further analysis. The ligation cocktail consisted of 5 µl buffer solution, one µl T4 DNA ligase, one µl pGEM-T Easy plasmid, and three µl AnsB gene amplicon. The total volume of the ligation cocktail was 10 µl. Furthermore, the ligation cocktail was incubated at 4 °C overnight. The recombinant plasmid was verified by PCR using the T7-SP6 primer provided in the plasmid genome using the protocol recommended by Promega. Transformation of the recombinant plasmid into the competent cell of E. coli strain DH5α was performed using the heat-shock protocol. The transformed cell was then propagated using LB-media and enriched for 1 hour at 37oC. The suspension subsequently propagated on LB-Agar containing ampicillin, IPTG and

XGal for blue-white selection. The recombinant cell was selected using PCR colony using T7/SP6 primer and, in some cases, using the Asnb-specific primer pair. Verification was determined.

Data processing and analysis

The data obtained at each stage were processed with thoroughness and presented visually, such as images, graphs, and diagrams. We utilized a range of bioinformatic applications and tools such as BLAST, MEGA X, Multiple Primer Analyzer, and OligoCalc for sequence homology analysis and primer designing. Our protein structure modeling was performed with the I-TASSER web tool (https://zhanggroup.org/I-TASSER/) and domain analysis using InterPro (https://www.ebi.ac.uk/interpro/), ensuring a robust and comprehensive analysis of our data.

RESULTS AND DISCUSSION

Quantity and Quality of isolated genomic DNA

Two replications (S1 and S2) of 1,5 µL suspension culture of *S. plymuthica* UBCF_13 produced significant DNA concentration. Electrophoresis analysis using 1% agarose exhibited high molecular weight DNA in a relatively similar amount compared to the λ-DNA used as reference (Figure 1). The result is consistent with the measurement of Bio-Drop using spectroscopy method 279 and 265 ng/µL for S1 and S2, respectively. Regarding its quality, Figure 1 exhibited a clear single main fragment indicating a DNA molecule with a large fragment size, and no extensive smear image was visible from both replications. This result is also consistent with the spectroscopy-based method as shown by Biodrop measurement, where the ratio of 260/280 for both samples is 1.898 and 1.792 for S1 and S2, respectively. A ratio of about 1.8 is generally accepted for DNA purity, while for RNA molecules should be at 2.0. If the ratio is lower or higher than 1.8, the DNA is considered to be contaminated with protein, phenol or other contaminants (Shah *et al*., 2019). This result clearly indicates the suitability of the protocol used in the DNA isolation of *S. plymuthica* UBCF_13, a gram-negative bacteria (Fatiah et al., 2021). This finding should instill confidence in the research methods. Therefore, the isolated DNA is ready to be used for further analysis. However, only S1 was used mainly for later downstream analysis.

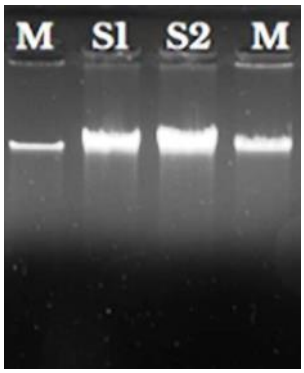


Figure 1. Electrophoresis image from sample 1 and 2 of isolated genomic DNA of *S. plymuthica* UBCF_13. M = λ DNA

Primer specific of AnsB Gene for S. plymuthica UBCF_13

A specific primer for gene isolation using a PCR-based method is a crucial step. For that reason, we designed a specific primer pair used for the isolation of the complete AnsB gene from *S. plymuthica* UBCF_13. Some characteristic of specific primer pairs for AsnB is shown in Table 3.

Table 3. Primer specific designed to amplify AnsB gene fragment.

Primer ID	Length (bp)	Sequence	GC%	Tm (°C)	*Self dimer*
Forward	23	5'- CCGGATGAAATCAGTGAAACTCA -3'	43.5	67.4	(-)
Reverse	23	5'- CCCCGGGGTCAATACTGATTAAA -3'	47.8	68.4	(-)

The specific primer, successfully designed at a length of 23 bp each, aligns with the optimal size range recommended by Borah (2011). This 23 bp length is considered ideal, as it falls within the 18 - 30 bp range suggested by Borah. Primers longer than 30 bp or shorter than 18 bp can compromise the specificity and efficiency of primer attachment to the DNA template. Additionally, a GC% value between 40 - 60% and a melting temperature (Tm) difference of no more than 5°C are also deemed suitable. An optimal GC proportion can enhance the stability of the primer-target DNA bond, thereby increasing the success of amplification in the PCR reaction.

Our designed primer sequences, AnsB_Forward and AnsB_Reverse, have successfully met all the criteria for an ideal primer. They exhibit no formation of self-dimers, cross-dimers, or hairpin structures, as any of these formations could potentially disrupt the PCR process (Purwakasih & Achyar, 2014). This achievement underscores the quality and reliability of our research.

The integrity of the designed primer pair was rigorously tested through a gradient temperature analysis, as detailed in the materials and methods. The results, presented in

Figure 2, show that the amplicon product, from G3 to G6, displayed a single clear band. This unequivocally demonstrates the effectiveness of the annealing temperature range in producing PCR product, instilling confidence in the reliability of our experimental results

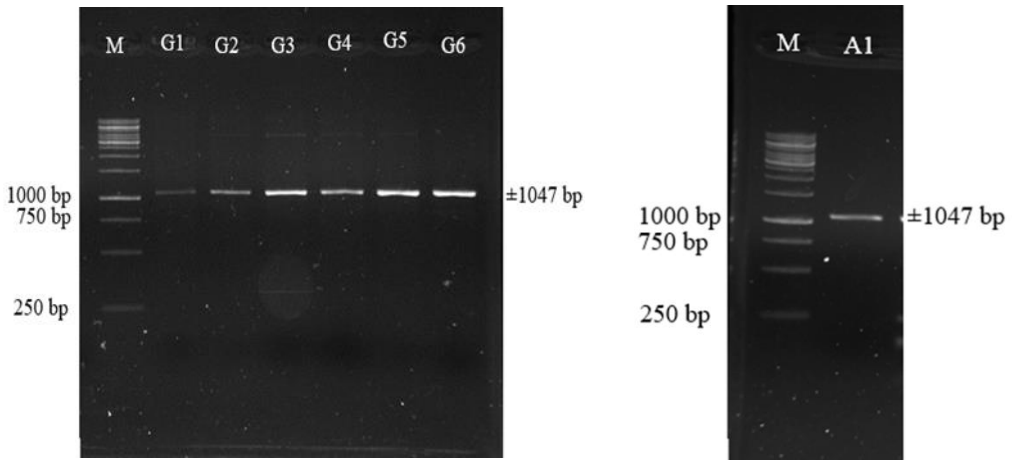


Figure 2. Gradient analysis of designed *ansB* primer pair. G1=48.2 ºC, G2=52.8 ºC, G3=57.4 ºC, G4=62 ºC, G5=66.6 ºC and G6=72 ºC (A). A single PCR product obtained using 65 ºC (A1) as annealing temperature. M = 1 kb size marker

Sequencing and bioinformatic analysis

To ensure the integrity and obtain sequence data from the amplicon produced by the AnsB-specific primer pair, bidirectional sequencing was performed using both primer pairs (forward and reverse). Raw data produced from sequencers were checked and edited for accuracy based on the quality of the electrophoregram (Rhalem et al., 2020). A crucial part of this process was the use of the BioEdit software (Hall, 1999), which was instrumental in eliminating any ambiguous nucleotide reads. After a series of trimming and editing, a single contig composed of 1,407 nucleotides was built and verified. The contig was started by the AsnB forward primer and ended with the AsnB reverse primer. The complete sequence data of the full length of the AsnB gene fragment is shown in Figure 3.

The accuracy of the AsnB gene fragment sequence data was verified through a rigorous process. We performed a BLAST homology analysis using FASTA data of the consensus nucleotide sequence against the nucleotide sequence available in the NCBI database. The BLAST result, which revealed that the accession with the highest homology (100%) is *Serratia plymuthica* UBCF_13, is a significant finding. This outcome confirms that the sequence of the AsnB gene fragment is indeed the true targeted gene. The genetic distance data produced among 10 selected available AnsB gene

sequences of *S. plymuthica* species, which can be used for phylogenetic tree construction using MegaX, further solidifies the accuracy of our findings (Smirnov & Warnow, 2021).

CCGGATGAAATCAGTGAAACTCA
ATGAAATCAGTGAAACTCAGCGTTGCGGCGCTACTCCTGGCGGGCGCGAGCGGTTCTGCGCTGGCATTGCCCTCCATCACTCTGTT
GGCAACTGGCGGCACCATTGCCGGCGGCGGAGAATCGGCGACAAAATCCAACTATACGGCGGGCAAACTGGGCGTCGAGCGCTG
GTGAATGCGGTGCCGGAGTTGAAAAACGTCGCCAATGTGCAGGGTGAGCAGGTAGTGAACATCGGTTCGCAGGATATGAACGATG
AGGTGTGGCTGAAACTGGTGAAGAAAATCAACGCCGACTGCGGCAAAACGGACGGCTTTGTGATCACCCACGGTACCGACACGC
TGGAAGAAACCGCCTATTTTCTCGATCTGACCGTCAAATGCGACAAGCCGGTGGTGATAGTCGGCGCGATGCGTCCGGCGACGGC
GATGAGCGCCGACGGCCCGTTCAATCTGTATAACGCCGTGGTGACGGCGGCGGATCCACAATCGGCCAAACGCGGGGTGCTGGTG
GCGATGAACGACAGCGTGCTGGATGCGCGCGACGTGACCAAAACCAATACCACCTCGGTGCAGACCTTCCAGTCGCCCAATTTCG
GCCCGCTCGGCTATATCCATAACGGCAAGGTCGACTATCAGCGTTCACCGCAGCGCAAGCATACTACCGATACCCCATTTGACGTAT
CCAGCCTGACCGAGCTGCCCAAGGTCGGCATTGTCTATAACTACGCCAACGCTTCCGATGCGCCCGCCAAGGCGCTGATCGCCGAA
GGTTATCAGGGGATCGTCAGCGCTGGGGTCGGTAACGGCAACCTGTACAAAACGGTGTTTGATACGTTGGCGACCGCCGCACATAA
CGGTGTGGCCGTGGTGCGCTCTTCAAGAGTGCCGACCGGTTCCACTACGGAAGACGCGGAAGTGGATGACGCTAAATTCGGTTTT
GTTGCCTCTGGCACGTTGAACCCGCAAAAAGCTCGGATTCTGCTGCAGTTGGCGCTGACTCAGACCAAAGACGCCAAACAGATCC
AACAGATGTTTAATCAGTATTGA
AAATTAGTCATAACTGGGGCCCC

Figure 3. Complete nucleotide sequence of *AsnB* gene isolated from *S. plymuthica* UBCF_13. Primer sequence forward and reverse are shown by shaded box in the left and right position

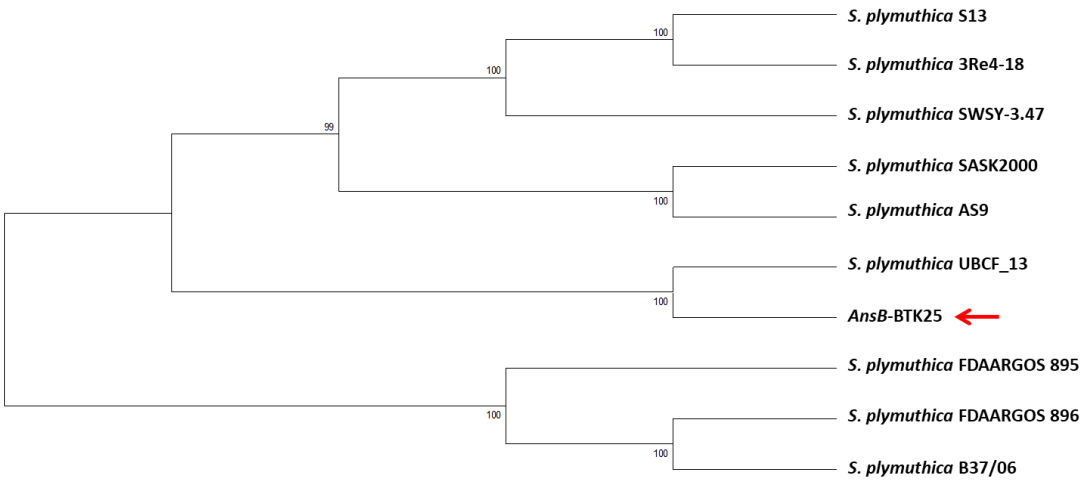


Figure 4. Cluster analysis showing position of the *AnsB* gene fragment (AsnB-BTK25 depicted with arrowhead) among other *Asn*B genes from 9 species of *S. plymuthica*

This study employed the efficient Neighbor-joining tree method to construct the phylogenetic trees. This method, which works by calculating the distance between each pair of sequences and selecting the pairs with the closest distance (or 'neighbors') to combine in the tree, is often used due to its simplicity, making it more efficient and faster in building phylogenetic trees (Azouri et al., 2021). According to Tallei et al. (2016), the smaller the genetic distance between two organisms, the closer the relationship between the two organisms. Based on the results of phylogenetic tree construction (Figure 4).

Figure 4 unmistakably demonstrates that the consensus sequence of the AsnB gene, derived from this study, clusters closely with the AsnB gene from *S. plymuthica*

UBCF_13 available in the NCBI database, with a value of 0.000 or 0%, confirming its originality. When compared to other AsnB genes, the consensus sequence from this study and *S. plymuthica* UBCF_13 cluster into different Output taxonomy units (OTU), indicating its unique characteristic. This unique characteristic of the AsnB gene sequence could potentially lead to a unique enzyme activity, which in turn could contribute to its specific catalytic activity. Further study on its enzyme activity is necessary to elucidate this possibility, although such analysis requires more extended time and more tedious work. As an alternative to such constraints, in Silico analysis of enzyme characteristics can be performed based on its nucleotide gene sequence.

In-silico domain analysis of L-asparaginase type 2 enzyme

Silico analysis used the I-TASSER and InterPro web tools (https://www.ebi.ac.uk/interpro/). The I-TASSER was used for structural modeling analysis, while the InterPro was used for protein domain analysis. The I-TASSER generated a large set of structural conformations, called decoys, using the SPICKER program to group all decoys based on the similarity of pair structure and provided five suitable models. The confidence of each model is measured quantitatively in the form of a C-score. The C-score value is usually in the range of -5 to 2, where a higher C-score value indicates a model with higher confidence and vice versa (Roy et al., 2010; Yang et al., 2015). The results of protein structure modeling (Table 5) show that model 1 has the highest and best confidence level among the other models, indicating a C-score value of -0.20. The next highest C-score was achieved by Model 2 (-0.56), followed by Model 4 (-0.70), then Model 5 (-1.25), and finally Model 3 (-1.30). Therefore, model 1 is the most representative model for the L-Asparaginase 2 enzyme from *S. plymuthica* UBCF_13. After tracing it to the PDB database, this protein structure is similar to the L-Asparaginase 2 from the *E. coli* strain Y25F, which Jaskólski et al. (2001) studied. In their study, Jaskólski et al. compared the crystal structure of two bacterial L-Asparaginases: the Y25F mutant from *E. coli* and the original L-Asparaginase from *Erwinia chrysanthemum*. These two enzymes show high sequence similarity (46% identity) but have different crystal packing.

Protein domain analysis, performed using the reliable InterPro web tool (https://www.ebi.ac.uk/interpro/), revealed that L-Asparaginase 2 from *S. plymuthica* UBCF_13 has two domains, namely L-Asparaginase, N-terminal located at 26th – 216th

its amino acid and Asparaginase/glutaminase, C-terminal which is located 235th – 345th aa. The C-terminal domain is smaller and connected by approximately 20 residues that form a linker. The N-terminal domain includes two threonine residues identified as playing a role in catalytic activity. One of the residues is located at the N-terminal end, while the other residue is located in approximately the first third of the sequence. The C-terminal domain is located at the C-terminal end of the asparaginase enzyme. This result underscores the effectiveness of InterPro in analyzing functional sites and specific domains, as described by Finn et al. (2017) and Paysan-Lafosse et al. (2023).

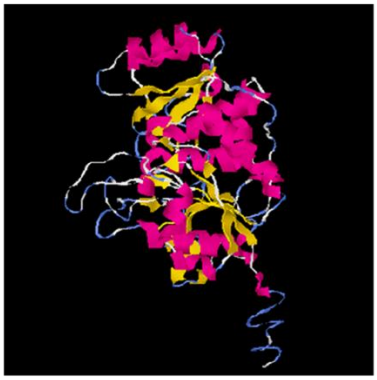
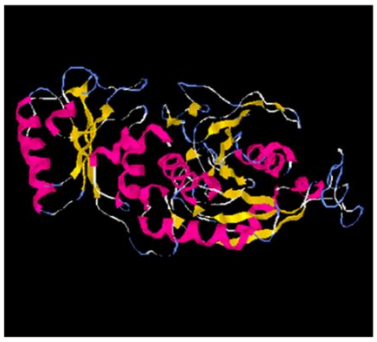
Model	3D-Structure	C-Score
#1		-0.20
#2		-0.56
#3		-0.70

Figure 5. Alternative 3D structure model of L-asparaginase type 2 enzyme from *S. plymuthica* UBCF_13

Ligation and transformation of AsnB gene fragment

The sustainability of physical AsnB gene fragments for further study is crucial. Therefore, we cloned the gene fragment into *E. coli* strain DH5α using pGEM T-Easy vector to preserve the gene fragment.

The resulting amplicon from the previous PCR product was used as material to be inserted into the pGEM-T Easy plasmid. After ligation was carried out by mixing the ligation components, the ligation cocktail was incubated for 16 hours at 4°C. The ligation result was verified using PCR by applying the T7/SP marker in the plasmid genome. Figure 6. shows a single amplicon fragment of about 1161 bp. The fragment length is summed up from the original amplicon, 1,047 bp, and the additional nucleotides amplified from the region flanked by both T7/SP6 primer pair, which is 219 bp.

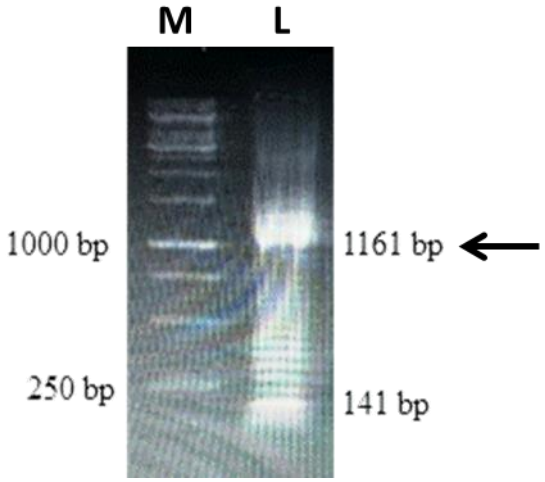


Figure 6. Verification of ligated the *asnB* gene fragment (L1) depicted by arrowhead in the pGEM T-Easy plasmid genome using T7/SP primer pair. M=1 kb size marker, L=ligated reaction

The reason for using T7-SP6 as a primer for this analysis is because the inserted gene fragment via the T-overhang mechanism provided in the pGEM T-Easy plasmid, which is located along the LacZ segment set as multi cloning site (MCS). The upstream part of the MCS contains the T7 promoter sequence, and the downstream part of the MCS is known as the SP6 promoter. The T7 and SP6 sequences can also be used as primer pairs for PCR analysis (Carter et al., 2022). Since the flanked T7/SP6 region of the pGEM T-Easy plasmid spanned 141 bp long, the amplicon using this primer pair should produce an additional result between 114 bp plus amplicon fragment successfully inserted in the

area. In this study, the AnsB gene fragment has a sequence size of 1047 bp. Thus, 114 added by 1047 is 1161 bp.

The recombinant pGEM-T Easy plasmid was transformed after successful ligation work into competent cell E. coli strain DH5α. The recombinant E. coli was selected based on its survival capability against ampicillin antibiotic added to the propagation LB-Agar Media. Moreover, the transformed recombinant plasmid containing gene target was verified using blue-white selection by adding IPTG and X-Gal in the propagation media (see material and method). Figure 7 shows transformed *E. coli* colonies on the selective LB-Agar media. Most white and some blue colonies are visible after overnight incubation of transformation suspension at 37oC. The presence of a single 1,047 bp fragment of the selected colony indicates the transformation process's success.

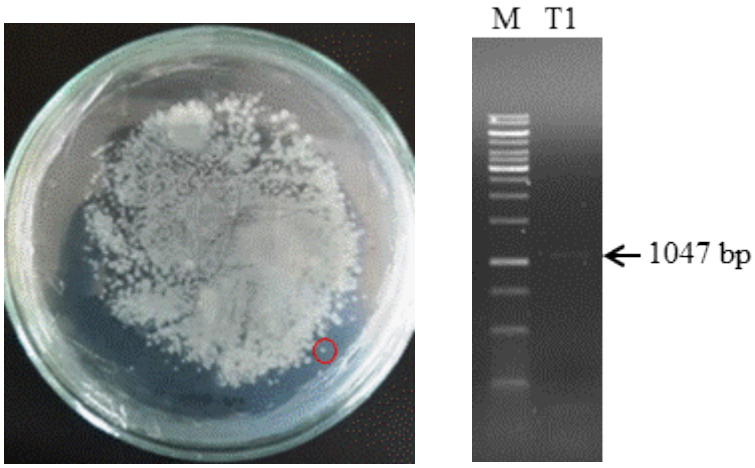


Figure 7. Blue white selection of transformed *E. coli* colony (A) and verification of the *AnsB* gene fragment using specific *AnsB* gene primer pair in the recombinant colony (T1) (B)

The blue colonies visible on the selective agar plate indicate that the transformant *E. coli* carries a recombinant plasmid resistant against Ampicillin. However, the blue colonies are regarded as empty plasmids since the integrity of the Lac-Z gene is still preserved. The blue color in the colonies is caused by the expression of β-galactosidase, which interacts with the X-gal compound, producing the blue pigment 5,5'-dibromo-4,4'-dichloro-indigo. The expression of β-galactosidase persists because the gene's coding region is not interrupted, indicating that the target gene is not inserted into the plasmid. On the other hand, the white colonies are regarded as the expected transformant since the Lac-Z gene has already been interrupted by the inserted AnsB gene fragment. The gene sequence encoding the β-galactosidase enzyme has been disrupted or interrupted in this

case. Since β-galactosidase is not appropriately expressed, the X-gal compound added in the media can not be hydrolyzed so that the colonies will produce a white color (Langley et al., 1975).

CONCLUSION

Based on the results described above, it can be confidently concluded that the isolation of the physical fragment of the AnsB gene is successfully done. Bioinformatic analysis indicated that the catalytic site of the L-asparaginase type enzyme from *S. plymuthica* strain UBCF_13 is located in the C terminal domain. Preserving the physical AnsB gene fragment is successfully using plasmid pGEM T-Easy vector hosted in the E. coli strain DH5α cell.

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